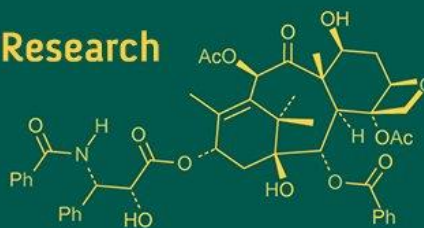


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## Genetic and phylogenetic analysis of Bluetongue virus serotype-2 isolate based on the outer capsid proteins VP2 and VP5

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### Abstract

Bluetongue virus (BTV) exhibits significant genetic diversity, with segment 2 (VP2) serving as the primary determinant of serotype and topotype classification. In contrast, segment 6 (VP5) is comparatively conserved and useful for understanding broader evolutionary relationships. In the present study, the genetic characterization of a BTV-2/CHN/IND isolate was performed based on nucleotide sequence analysis of the VP2 and VP5 genes. The phylogenetic analysis of BTV-2 based on outer capsid protein segment-2 (VP2) and segment-6 (VP5) reveals that BTV-2/CHN/IND isolate is more closely related to American, South African, and European strains (Western topotypes) than Asian strains (Eastern topotypes). Percent identity analysis of the VP2 gene and VP5 gene revealed that the isolate shares very high nucleotide identity (99.5-99.8%) with BTV-2 strains from South Africa, Spain, and Israel, indicating close genetic relatedness. In contrast, a lower percent identity was observed with isolates from Australia, Taiwan, and earlier Asian strains. These findings clearly positioned the BTV-2/CHN/IND isolate within the Western topotype, despite its geographic association with Asia.

**Keywords:** Bluetongue, diversity, serotype, topotype, nucleotide sequence

### Introduction

Bluetongue (BT) is an infectious, non-contagious, and economically important hemorrhagic disease of domestic and wild ruminants, which is transmitted by *Culicoides* midges. BT is caused by the bluetongue virus (BTV), a member of the genus *Orbivirus* in the family *Sedoreoviridae* (ICTV taxonomy release 2025). Sheep are the most susceptible host, together with white-tailed deer, bighorn sheep, and pronghorn antelope, causing clinical disease. Subclinical manifestation is seen in cattle, buffalo, and goats, which serve as reservoirs of the virus. (Rupner *et al.*, 2020; Saminathan *et al.*, 2020; Balwan *et al.*, 2021) [23, 24, 2]. The first outbreak of BT occurred in South Africa in the late 18th century, while in India, BTV was first reported in 1964 in the state of Maharashtra based on clinical signs and detection of anti-bluetongue antibodies (Sapre, 1964) [25]. Bluetongue is considered an emerging disease and is listed by the World Organisation for Animal Health (OIE) as a 'Notifiable Disease' (OIE, 2019). Currently, 29 serotypes are reported worldwide, including the atypical serotypes BTV-25, 26, and 27 (Hofmann *et al.*, 2008; Maan *et al.*, 2011; Zientara *et al.*, 2014) [6, 14, 29] based on serotype-specific virus neutralization assays (VNTs) and VP2 sequence analysis (Lakshmi *et al.*, 2018; Kalyani *et al.*, 2019) [12, 10]. The atypical serotypes of BTV, such as BTV 25 (Toggenburg virus strain) detected in healthy goats of Switzerland in 2007, BTV 26 identified in sheep of Kuwait in 2010 and BTV 27 isolated in Corsica of France from goats in 2014 (Maan *et al.*, 2011; Jenckel *et al.*, 2015) [14, 9]. Virus neutralization tests (VNT), VP2 gene sequencing, and reverse transcription polymerase chain reaction (RT-PCR) are instrumental in identifying BTV serotypes (Maan *et al.*, 2017) [16].

Bluetongue Virus is non-enveloped, linear with icosahedral symmetry. The BTV genome comprises 10 double-stranded RNA (dsRNA) segments, which encode seven structural proteins (VP1 through VP7) and five non-structural proteins (NS1, NS2, NS3/NS3A, NS4, and NS5). The total length of the BTV genome is approximately 19.2 kbp, with individual dsRNA segments ranging from 3954 to 822 bp in length, ordered by decreasing molecular weight (from Segment 1 to Segment 10) (Roy *et al.*, 1990; Balwan *et al.*, 2021) [22, 2].

Three concentric capsid layers are present in the mature BTV virion. Segment 2 encodes 60 trimers of VP2 (110 kDa) and Segment 6 encodes 120 trimers of VP5 (59 kDa) (Mertens *et al.*, 1989; Mertens and Diprose 2004) <sup>[18, 19]</sup>. It is the primary determinant of the BTV serotype and is essential for triggering hemagglutination and neutralizing antibodies which is unique to the serotype. The intermediate capsid layer, composed of 260 trimers of VP7 (38 kDa, encoded by Segment 7), is highly conserved region and the key determinant of BTV serogroup (Mertens and Diprose 2004; Hewat *et al.*, 1994) <sup>[19, 5]</sup>. The variations in the sequence of nucleotide of BTV strains known as "BTV topotypes" correspond with their geographical regions or origins, and can be further subdivided into groups based on their origin, significant phenotypic and genotypic differences (Bonneau *et al.*, 2001; Shaw *et al.*, 2013) <sup>[3, 26]</sup>. According to their geographic origin, the majority of BTV's genome segments can be clearly classified into two topotypes or groups: 'Eastern' (BTV originated from Asia which includes Indian subcontinent, Japan, East Asia-China, Taiwan, Southeast Asia Indonesia), Europe, Australia, Middle East (Turkey), and Mediterranean Basin, and 'Western' (African continent like South Africa and Nigeria), USA (Brazil and Guatemala), Caribbean region namely Jamaica and Caribbean islands. BTV strains have developed over an extended period with genetic exchange between regions and several point mutations, enabling them to acquire distinct regional distinctions (Maan *et al.*, 2010) <sup>[15]</sup>.

## Materials and Methods

- a. **Cells and virus isolate:** The BHK-21 cell line was revived in 10% growth media and incubated at 37°C. When the monolayer was completed, cells were subcultured to a new cell culture flask. Virus isolate BTV-2/CHN/IND was revived on BHK-21 cell line maintained at the virus repository at the ICAR-Indian Veterinary Research Institute (IVRI), Virology division, Mukteswar, Uttarakhand. The infected flask was monitored for cytopathic changes (CPE). After 80-90% CPE, infected cells were harvested and propagated further in a 75cc cell culture flask.
- b. **Extraction of viral dsRNA:** Total RNA of BTV-2/CHN/IND was extracted from virus-infected cells using the Trizol method and purified by lithium chloride precipitation (Attoui *et al.*, 2000) <sup>[1]</sup>. The virus-infected cell lysate was transferred from the 75 cc cell culture flask to a 15 ml centrifuge tube and centrifuged at 2000 rpm for 10 minutes. Supernatant was discarded, and pellet was suspended in a resuspension buffer that contains lysis buffers like Tris-EDTA, 10% SDS, 3M sodium acetate (pH 4.5), and proteinase K. The cell pellet with the resuspension buffer was incubated at 47°C for 1 hour. The cell lysate was then used for RNA extraction by TRI-reagent (Sigma) according to the manufacturer's protocol. Viral dsRNA was precipitated sequentially with 4 M LiCl and then 8 M LiCl, and eluted in TAE buffer. The 10 segments of the BTV virus were analysed in 2% agarose gel electrophoresis.
- c. **cDNA synthesis and PCR amplification of the VP2 gene:** After the visualization of the 10 segments, segment 2 was excised from the gel and purified using a gel purification kit (QIAquick®, Qiagen) according to the manufacturer's protocol. The gel-purified product

was used for PCR amplification with the BTV-2 primer targeting the VP2 gene.

- d. **Sequencing and Phylogenetic analysis of the full genome sequence of BTV-2 serotype:** The quality and quantity of the extracted dsRNA were assessed using a Nanodrop, and the dsRNA was outsourced for sequencing to obtain full-genome sequence data via next-generation sequencing (NGS) on the Ion Torrent platform. The nucleotide sequence data for the VP2 gene were assembled to obtain the complete coding sequence. The identity of the sequence was confirmed by NCBI BLASTN analysis. The length of each genomic segment was calculated using a command-line approach in a Linux terminal environment, and the coding sequences (ORFs) of the genomic segments were analyzed using the ORF finder provided by the National Center for Biotechnology Information (NCBI). Reference sequences of BTV-2 were retrieved from GenBank, and multiple alignments were performed using the CLUSTAL W algorithm (Thompson *et al.*, 1994). The sequences were analyzed for possible recombination breakpoints and potential intragenic recombination events using Recombination Detection Programme 4 (RDP4) (Martin *et al.*, 2015) <sup>[17]</sup> and SimPlot 3.5.1 (Lole *et al.*, 1999) <sup>[13]</sup>. Percentage identity calculations and phylogenetic tree construction were performed using MEGA version 6.0 software (Tamura *et al.*, 2013) <sup>[27]</sup>.

## Results

The length of segment 2, which codes for the outer capsid protein VP2 gene, was found to be 2943 bp in length, and the complete coding region was 2889 nucleotides long (including the start and stop codon), encoding for 962 amino acids. The length of segment 6 which codes for the outer capsid protein VP5 gene, was 1635 bp, with a coding region of 1572 nucleotides encoding 523 amino acids. Phylogenetic analysis based on VP2 gene revealed that BTV-2/CHN/isolate clustered with BTV-2 of Indian isolates (KP696593 and KP696603), along with isolates from RSA (JN255923, JN55933) (Wild type and Vacc), USA (M21946), Spain (KP821035) and ISR (KP821027) as shown in Fig 1. (A). BTV-2/CHN/IND isolate showed the highest nucleotide percentage identity of 99.7 to 99.9% with reference strains from South Africa (SA), USA, Spain, Israel and Indian isolates indicating close genetic relatedness and shared lineage. A lower percent identity of approximately 88-90% was observed with recent South African isolates from 2020, suggesting genetic divergence between them. (Table: 1)

Phylogenetic analysis based on the VP5 gene clusters tightly, having short branch length with RSA (JN255927, JN55937) (wild type and vaccine), SPA (KP821517), ISR (KP821509), and Indian isolate (AJ783905), while with the AUS (JQ240326), TWN (AY493690), and IND(AJ586675) isolates have long branch lengths (Fig.1.B). BTV-2/CHN/IND isolate exhibits a high percent identity of 99.5 to 100% with Spain, Russia, Israel, and India, moderate identity of 87 to 88% with USA, South Africa, Sudan, and France, and low identity with Taiwan, Australia, and India (old isolates from 1982) (Table:2)

There was no evidence of any intragenic recombination event in the sequence of the BTV-2/CHN/IND isolate, as revealed by RDP4 and the Simplot software package.

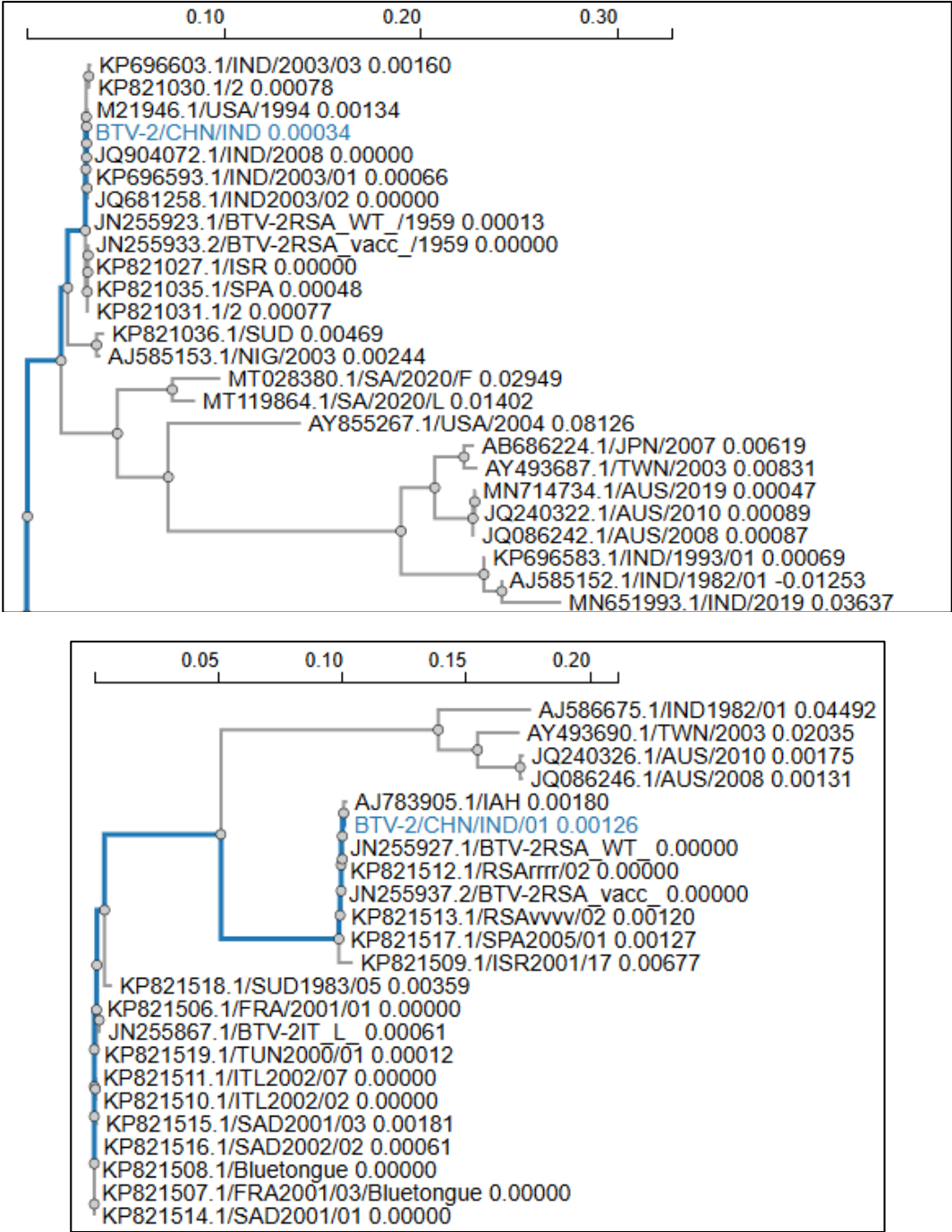


**Fig 1:** Phylogenetic tree of the full genome sequence of BTV-2/CHN/IND constructed with CLUSTAL Omega depicting the genetic relation of BTV-2/CHN/IND with other global BTV-2 isolates based on the outer capsid protein (a) VP2 gene and (b) VP5 gene, where BTV-2/CHN/IND is seen to cluster more tightly with Western isolate

**Table 1:** Percent Identity matrix of BTV-2/CHN/IND (in bold) with other global isolates based on segment 2 (VP2 gene).

|    |                           |       |       |       |       |       |       |       |       |        |       |       |       |       |       |    |
|----|---------------------------|-------|-------|-------|-------|-------|-------|-------|-------|--------|-------|-------|-------|-------|-------|----|
| 1  | AJ586675.1/IND1982/01     | ID    |       |       |       |       |       |       |       |        |       |       |       |       |       |    |
| 2  | AY493690.1/TWN/2003       | 91.74 | ID    |       |       |       |       |       |       |        |       |       |       |       |       |    |
| 3  | JQ240326.1/AUS/2010       | 91.13 | 95.72 | ID    |       |       |       |       |       |        |       |       |       |       |       |    |
| 4  | JQ086246.1/AUS/2008       | 91.30 | 95.77 | 99.69 | ID    |       |       |       |       |        |       |       |       |       |       |    |
| 5  | AJ783905.1/IAH            | 79.02 | 79.14 | 79.57 | 79.61 | ID    |       |       |       |        |       |       |       |       |       |    |
| 6  | BTV-2/CHN/IND/01          | 78.96 | 79.08 | 79.51 | 79.55 | 99.69 | ID    |       |       |        |       |       |       |       |       |    |
| 7  | KP821517.1/SPA2005/01     | 79.27 | 79.27 | 79.69 | 79.73 | 99.51 | 99.57 | ID    |       |        |       |       |       |       |       |    |
| 8  | KP821513.1/RSAvvvv/02     | 79.14 | 79.33 | 79.76 | 79.79 | 99.57 | 99.63 | 99.69 | ID    |        |       |       |       |       |       |    |
| 9  | JN255927.1/BTV-2RSAWT     | 79.14 | 79.27 | 79.69 | 79.73 | 99.76 | 99.82 | 99.76 | 99.82 | ID     |       |       |       |       |       |    |
| 10 | KP821509.1/ISR2001/17     | 78.59 | 78.78 | 79.14 | 79.18 | 98.96 | 99.02 | 99.08 | 99.14 | 99.20  | ID    |       |       |       |       |    |
| 11 | KP821512.1/RSArtrr/02     | 79.14 | 79.27 | 79.69 | 79.73 | 99.76 | 99.82 | 99.76 | 99.82 | 100.00 | 99.20 | ID    |       |       |       |    |
| 12 | JN255937.2/BTV-2RSA_vacc_ | 79.20 | 79.33 | 79.76 | 79.79 | 99.69 | 99.76 | 99.82 | 99.88 | 99.94  | 99.27 | 99.94 | ID    |       |       |    |
| 13 | KP821506.1/FRA/2001/01    | 78.84 | 79.69 | 79.08 | 79.12 | 87.77 | 87.83 | 88.01 | 87.95 | 88.01  | 87.52 | 88.01 | 88.07 | ID    |       |    |
| 14 | KP821518.1/SUD1983/05     | 78.96 | 79.82 | 79.08 | 79.12 | 87.89 | 87.95 | 88.13 | 88.07 | 88.13  | 87.65 | 88.13 | 88.20 | 99.14 | ID    |    |
| 15 | KP821515.1/SAD2001/03     | 78.72 | 79.57 | 78.96 | 79.00 | 87.52 | 87.58 | 87.77 | 87.71 | 87.77  | 87.28 | 87.77 | 87.83 | 99.57 | 98.96 | ID |

**Table 2:** Percent Identity matrix of BTV-2/CHN/IND with other global isolates based on segment 6 (VP5 gene).

|                                   |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |    |
|-----------------------------------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|----|
| 1: DQ191275.1/ITL/2005            | 87.97 | 89.67 | 96.3  | 96.26 | 96.33 | 96.26 | 96.19 | 96.16 | 96.19 | 96.16 | 95.85 | 95.41 | 95.17 | 99.93 | 99.97 | 99.97 | ID |
| 2: KP821025.1/FRA                 | 88.01 | 89.7  | 96.33 | 96.3  | 96.36 | 96.23 | 96.23 | 96.19 | 96.13 | 95.75 | 95.14 | 95.45 | 95.21 | 99.97 | 100   | ID    |    |
| 3: KP821026.1/FRA                 | 88.01 | 89.7  | 96.33 | 96.3  | 96.36 | 96.3  | 96.23 | 96.19 | 96.23 | 96.23 | 95.89 | 96.13 | 95.14 | 99.97 | ID    |       |    |
| 4: KP821034.1/SAD                 | 88.01 | 89.7  | 96.33 | 96.36 | 96.23 | 96.19 | 96.23 | 96.19 | 96.02 | 96.13 | 95.75 | 95.21 | 99.97 | ID    |       |       |    |
| 5: KP821032.1/SAD                 | 87.97 | 89.67 | 96.3  | 96.26 | 96.33 | 96.26 | 96.19 | 95.99 | 96.09 | 95.11 | 95.41 | 95.17 | ID    |       |       |       |    |
| 6: KP696603.1/IND/2003/03         | 88.04 | 88.74 | 99.69 | 99.66 | 99.66 | 99.66 | 99.59 | 99.56 | 99.39 | 99.46 | 99.86 | ID    |       |       |       |       |    |
| 7: KP821027.1/ISR                 | 88.45 | 90.15 | 99.76 | 99.73 | 99.8  | 99.73 | 99.69 | 99.63 | 99.73 | 99.83 | ID    |       |       |       |       |       |    |
| 8: JN255933.2/BTV-2RSA_vacc_/1959 | 88.45 | 90.15 | 99.76 | 99.73 | 99.8  | 99.73 | 99.69 | 99.63 | 99.93 | ID    |       |       |       |       |       |       |    |
| 9: KP821035.1/SPA                 | 88.38 | 90.08 | 99.69 | 99.66 | 99.73 | 99.66 | 99.63 | 99.56 | ID    |       |       |       |       |       |       |       |    |
| 10: M21946.1/USA/1994             | 88.21 | 89.91 | 99.86 | 99.83 | 99.83 | 99.83 | 99.76 | ID    |       |       |       |       |       |       |       |       |    |
| 11: KP696593.1/IND/2003/01        | 88.28 | 89.98 | 99.9  | 99.93 | 99.86 | 99.86 | ID    |       |       |       |       |       |       |       |       |       |    |
| 12: BTV-2/CHN/IND                 | 88.31 | 90.01 | 99.97 | 99.93 | 99.93 | ID    |       |       |       |       |       |       |       |       |       |       |    |
| 13: JN255923.1/BTV-2RSA_WT_/1959  | 88.38 | 90.08 | 99.97 | 99.93 | ID    |       |       |       |       |       |       |       |       |       |       |       |    |
| 14: JQ681258.1/IND2003/02         | 88.31 | 90.01 | 99.97 | ID    |       |       |       |       |       |       |       |       |       |       |       |       |    |
| 15: JQ904072.1/IND/2008           | 88.35 | 90.04 | ID    |       |       |       |       |       |       |       |       |       |       |       |       |       |    |
| 16: MT119864.1/SA/2020/L          | 95.62 | ID    |       |       |       |       |       |       |       |       |       |       |       |       |       |       |    |
| 17: MT028380.1/SA/2020/F          | ID    |       |       |       |       |       |       |       |       |       |       |       |       |       |       |       |    |

## Discussion

Reddy Y.K. and colleagues in 2016 sequenced BTV-2 isolates from the 2003-2011 outbreak and found that BTV-2 isolates from Tamil Nadu were identical to sequences from Africa and the USA (Reddy *et al.*, 2016). The full-length genome sequencing of segment 5 (NS1) of the Indian isolate VJW64 (BTV-16) showed >89.5% nucleotide identity with Western topotype strains and <78% identity with Eastern isolates, clustering within the Western lineage. The findings provide evidence for the circulation of a reassortant BTV-16 strain in India. (Pawan. P *et al.*, 2013) <sup>[11]</sup>. There are a few studies that have revealed the event of reassortment between serotypes. The Western and Eastern topotypes form two separate clades in the phylogenetic tree. BTV-2/CHN/IND isolates cluster with African, European, and American strains within the Western topotype, as well as with Indian isolates. Analysis of segment 2 (VP2) gene and segment 6 (VP5) gene BTV-2/CHN/IND isolate was compared with several strains belonging to the global reference, which revealed very high nucleotide percent identity, that is, 99.6 to 99.9% with western topotypes like South Africa (RSA wild-type and vaccine strains), Israel, Spain, the United States, and previously reported Indian isolates indicating close genetic relatedness. Moderate identity (87-88%) with other global isolates suggests intra-lineage variation within the Western topotype. Whereas lower nucleotide identity of 72 to 73% were observed with isolates from East and Southeast Asia (China, Australia, Taiwan, and Japan), which were Eastern topotypes, indicating genetic divergence. These findings confirm the isolate's classification within the Western lineage.

## Conclusion

Phylogenetic clustering and percent identity analysis are highly concordant, providing robust evidence that BTV-2/CHN/IND belongs to the Western topotype. These findings highlight the dynamic nature of BTV evolution and underscore the importance of continuous molecular surveillance to monitor lineage distribution, reassortment events, and the potential emergence of novel variants. Short branch lengths among Western strains reflect their high nucleotide similarity, whereas a long genetic distance separates them from the Eastern topotype. This divergence between geographic origin and genetic lineage suggests potential transboundary introduction of Western lineage viruses into Asian regions. Such movement may occur

through vector dispersal, animal trade, or ecological changes influencing vector distribution.

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